Archivum Immunologiae et Therapiae Experimentalis, 2000, **48**, 225–233
PL ISSN 0004-069X

Review

Differential Effects of CD40 Stimulation on Normal and Neoplastic Cell Growth

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Abstract. CD40 is a molecule in the tumor necrosis factor receptor/nerve growth factor receptor (TNFR/NGFR) family that is present on both normal and neoplastic B lineage cells. It is also expressed on carcinoma and melanoma cells and can be augmented with interferon γ. CD40 stimulation in normal B cells has been demonstrated to promote normal B cell differentiation and growth *in vitro*. In contrast to these effects, CD40 stimulation by either anti-CD40 antibodies or a recombinant soluble CD40 ligand can inhibit the growth of human breast carcinomas and aggressive histology B lymphomas *in vitro* and *in vivo*. This is believed to occur by activation--induced cell death (AICD) in which stimuli that promote the growth of normal cell types inhibit the growth of neoplastic counterparts. This occurs through the induction of apoptosis, necrosis and/or cell cycle arrest. Thus, CD40 stimulation may be of potential clinical use in the treatment of carcinomas and B cell lymphomas. This review shall provide an overview of the various effects of CD40 stimulation on both normal and neoplastic cell types.

Key words: CD40; apoptosis; transformed; lymphoma; interferon γ.

CD40

CD40 is a 55 kDa molecule present on a variety of cell types ranging from B cells[9] to endothelial cells[30], dendritic cells[33], some carcinomas[24], lymphomas[12], melanomas[48] and hematopoietic progenitor cells. CD40 is a type 1 membrane glycoprotein[33]. It has 277 amino acids with four similar repeating cysteine-rich extracellular domains, each approximately 45 amino acids long[44]. It is a member of the nerve growth factor (NGF)/tumor necrosis factor receptor (TNFR) family which includes CD30 and CD95 (Apo-1/Fas)[44].

CD40 appears to play a critical role in the growth and differentiation of untransformed B cells[3, 9, 12]. CD40 activation is required for antigen stimulated B cells to produce specific isotypes of antibodies (IgG, IgA, IgM and IgE) in response to various cytokines, as well as proliferation and isotype switching[33, 38]. B cells are able to proliferate and survive long-term in culture when CD40 is activated along with IL-4 *in vitro*[7]. Li-

This project has been funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health under contract no. N01-CO-56000.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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gation of CD40 activates resting B cells while increasing their surface expression of stimulatory molecules that are involved in heterotypic aggregation[33]. B cell immunoglobulin (Ig) receptor activation and CD40 stimulation leads to phenotypic (CD38 and Fas) as well as functional changes in germinal centers of the lymph node, within which CD40 stimulation causes B cells to differentiate into memory cells instead of plasma cells[4]. Ligation of CD40 on dendritic cells and monocytes increases cytokine production and expression of costimulatory molecules[33].

It has been shown that members of the TNFR family associate with various signaling pathways such as the TNFR-associated factor (TRAF) family and the Ice caspase family[33]. In contrast to the TNFR and Fas families, CD40 does not contain a "death domain" (DD) in the cytoplasmic tail[44]. Although CD40 lacks a DD, there is a shared homology with the intracellular region of p55 TNFR[27, 47]. The most prominent protein associated with CD40 signaling is TRAF3[26]. Although the precise role for this protein is not known, it has a noticeable association with the CD40 cytoplasmic tail in yeast two-hybrid experiments[43]. TRAF1 and TRAF2 contain specific DNA-binding protein motifs that could possibly act as transcriptional regulators[43]. TRAF2 is responsible for the activation of NF-κB through CD40[42].

CD40 Ligand

CD40 ligand (CD40L): gp39, CD154, is a wide pleiotropic agent, affecting a large repertoire of cellular immunology. Activation of CD40 requires contact with its ligand CD40L, a 39 kDa type 2 membrane glycoprotein that is primarily found on activated $CD4^+$ T lymphocytes[25], mast cells and basophils[2, 35]. CD40L has also been detected on blood dendritic cells[40], eosinophils[16], activated NK cells[8], B cells[19] and platelets[22]. Individuals congenitally lacking this ligand have a deficiency in circulating IgG antibodies, also known as hyper IgM syndrome[1]. This syndrome is characterized by a decrease in antibody production and defective antibody class switching[1].

CD40L is a member of the TNF family[25]. The gene maps to the q26 region of the X chromosome[18]. The protein contains 261 amino acids, with a 215 amino acid extracellular domain, a 24 amino acid transmembrane domain, and a 22 amino acid intracellular domain[25]. CD40L induces cytokine production, regulates B cell function and exhibits tumoricidal activity in peripheral blood monocytes[2, 3, 48]. Recombinant CD40L in the presence of interleukin 4 (IL-4) and IL-13 causes the proliferation of B cells and isotype switching[6].

CD40L has been engineered to exist as a soluble ligand[18]. The soluble recombinant ligand contains the extracellular domain of CD40L fused to an amino-proximal 30 amino acid-modified leucine zipper motif[35]. Murine and human forms of CD40L have been shown to be directly mitogenic for murine and human B lymphocytes *in vitro*[2, 3]. In addition to the induction of proliferation and differentiation on B cells, srCD40L has also been shown to prevent apoptosis of B cells[35]. Furthermore, CD40L has also been shown to be an important costimulatory molecule present on T cells[3]. Even though CD40L is expressed mainly on $CD4^+$ T cell populations, the ligand has been shown to exert a stimulatory function on both $CD4^+$ and $CD8^+$ T cell populations[3]. Engagement of CD40 by CD40L on activated $CD4^+$ T cells is believed to result in the trimerization of CD40 on B cells and is thought to lead to signal transduction[5]. CD40-CD40L interactions thus play critical roles in immune function.

CD40 Expression and Function on Neoplastic Cells

CD40 has been shown to be expressed at high levels on various types of lymphomas, including non-Hodgkin's lymphomas (NHL)[51], follicular non-Hodgkin's lymphomas (F-NHL)[29, 50], hairy-cell leukemia[32], and B chronic leukocytic leukemia (B-CLL)[10]. Stimulation of CD40 on these neoplasms gives rise to different results depending on the particular tumor type. Activation of CD40 on hairy cell leukemias, F-NHL, B-CLL, and indolent (low-grade, slow progressing) neoplastic B cells has been shown to promote growth[5, 10, 29, 32, 33]. In marked contrast, high-grade aggressive histology NHL such as Burkitt's lymphoma and Epstein-Barr virus (EBV)-derived lymphomas do not proliferate when stimulated by CD40[15]. Stimulation of CD40 in Burkitt's lymphoma and EBV lymphomas inhibits cell growth presumably by the induction of activation-induced cell death (AICD)[12, 13, 15].

Myeloma is a neoplasm of terminally differentiated bone marrow plasma cells[39, 49]. CD40 is present on the majority of established myeloma cell lines and myeloma cells isolated from plasma cell dyscrasia (PCD) patients[49]. Interestingly, malignant plasma cells have increased CD40 expression over untransformed plasma cells[49]. CD40 stimulation by either anti-CD40 antibody or CD40L modulated growth in these neoplasms[49]. It was found that treatment with anti-CD40 antibodies

promoted clonogenic growth and enhanced *in vitro* growth[49]. However, it has also been shown that myeloma lines highly expressing CD40 are completely growth inhibited by CD40 stimulation[39]. This growth inhibition occurred by incubation with either CD40L or anti-CD40 antibody[39].

CD40 is also present on some melanoma and carcinoma cells, although to a much lesser extent than on lymphoma cells[24, 48]. Human malignant melanoma (MM) is an immunogenic tumor that is infiltrated by $CD4^+$ and $CD8^+$ T cells[41]. Stimulation of CD40 on MM's has been shown to induce apoptosis as well as enhance tumor-specific cytotoxic T lymphocyte (CTL)--mediated killing of these cells[34, 47]. CD40 ligation on established melanoma cells lines stimulates the release of IL-6, IL-8 and TNF-α[34, 47].

CD40 is also found on carcinomas of the breast[45, 52], and bladder[28], although present to a much lesser extent on their normal tissue counterparts. The functional significance of CD40 on these transformed cells is not yet known. WINGETT et al.[52] showed that CD40 stimulation on breast carcinomas results in enhanced susceptibility to Fas-mediated apoptosis and marked growth inhibition. Similarly, stimulation of CD40 on breast carcinomas by CD40L was shown to result in AICD and a decrease in proliferation[24, 52]. Interestingly, while CD40 is present on the normal tissue counterpart of B cell lymphomas, it is not expressed on normal breast epithelial cells to a significant degree[45]. This surprising observation implies a potential role of CD40 in the survival and progression of these neoplasms.

IFN-γ Increases CD40 Expression on Carcinoma Lines

Treatment of several human breast carcinoma lines with IFN-γ resulted in increases in the surface expression of CD40, suggesting potential adjunct use in tumor therapy[9, 24]. This treatment can also augment the growth inhibitory effects of CD40L *in vitro*[9, 24]. Four breast carcinoma lines were used by HIRANO et al.[24] for studies with IFN-γ and CD40L. MDA231 cells were isolated from a poorly differentiated adenocarcinoma, BT20 cells from an infiltrating ductal carcinoma, and T47D cells from a pleural effusion of a ductal carcinoma[24]. Each of these lines was pretreated with 500 U/ml IFN-γ prior to CD40 expression staining or anti-CD40 treatment. HIRANO et al.[24] showed that pretreatment with IFN-γ can increase CD40 expression on human breast carcinoma lines (Table 1), and increased growth inhibition of these lines. Thus, the subsequent increase in CD40 expression following IFN-γ treatment renders these neoplastic cells more susceptible to inhibition by CD40L therapy. Furthermore, WINGETT et al.[52] also showed that the levels of CD40 on MDA231, T47D and BT20 carcinoma lines are also increased with IFN-γ treatment.

Table 1. The effects of IFN-γ treatment on CD40 expression in human breast carcinoma

Cell line	CD40 expression without IFN-γ (%)	CD40 expression with IFN-γ 100 U/ml, 48 h (%)
Daudi	80.6	–
MDA231	24.0	81
T47D	10.2	51.2

Daudi, MDA231, and T47D cells were analyzed for changes in CD40 expression due to IFN-γ stimulation. Cells were stimulated with 100 U/ml IFN-γ for 48 h. A control group was included, with no IFN-γ stimulation. Cells were stained with anti-CD40 (M2 clone), followed by a secondary stain of goat-anti-mouse-fluoroisothiocyanate (GAM-FITC). The cells were then analyzed on an EPICS flow cytometer.

Activation-Induced Cell Death

It has been shown that some signaling agents of normal cell activation can inhibit the growth of transformed cells. This phenomenon is known as AICD and has been extensively studied in lymphocytes. AICD can occur by cell cycle arrest, apoptosis and/or necrosis[17, 21, 33]. Examples of AICD are: cell cycle arrest by anti-CD19[17], and CD30 mediated necrosis[21]. Along with these mechanisms, members of the TNF family such as Fas/APO-1, contain receptors also known as DD[20] that signal cell death upon ligation. Engagement of these receptors often causes the induction of caspases, therefore causing the cell to undergo apoptosis[31]. Stimulation of CD30 was also shown to induce death on large cell lymphomas and cells of epithelial origin[23]. CTL are also able to eradicate their target cells through these death receptors[23]. GRELL et al.[20] showed that cytotoxic effects induced by CD30, CD40 and TNFR-2 are mediated by the exogenous production of TNF, there by causing an activation of TNFR-1 and an increase in cytotoxicity.

Somewhat paradoxically, CD40 has been shown to be an anti-apoptotic molecule, as it has the ability to rescue untransformed B lymphocytes and low grade lymphomas from apoptosis[28]. However, CD40 stimulation has been shown to cause AICD in some aggressive B lymphomas and carcinomas[17]. The mechanism by

which this occurs is yet unknown, since CD40 contains no DD. Possible mechanisms include Fas triggering and the induction of various cytokines such as TNF-α and appear to be related to the neoplastic state of the tumor[20, 33].

CD40 Stimulation as a Therapy for Lymphoma and Carcinoma

The use of anti-CD40 antibodies *in vitro* as well as *in vivo* has proved to be inhibitory against various human carcinomas and lymphomas. CD40 specific antibodies significantly inhibit the proliferation of Burkitt's lymphoma lines as well as lines from a diffuse large cell lymphomas[12]. An optimal growth inhibition of 40–60% in the Daudi Burkitt's lymphoma line (as assessed by thymidine incorporation) was reached with anti-CD40 antibodies (M2 and M3 hybridomas, IgG1)[12, 13]. In examining the effects of anti-CD40 on lymphoma growth *in vitro* it was found that cross-linking of the antibody significantly increased the inhibitory effects seen in these lines in contrast to treatment with a soluble antibody[12]. For example, soluble anti--CD40 monoclonal antibodies (mAbs) showed no inhibitory effects *in vitro* on the Burkitt's lymphoma line Raji; however, prior immobilization of the antibody did result in inhibition[15, 13]. Similar effects on cell growth were also seen when CD40 was stimulated in human breast carcinomas (Fig. 1). Anti-CD40 antibodies have been shown to prevent human B cell lymphomagenesis in the HuPBL/SCID mouse model while concurrently promoting human B cell engraftment[12, 15]. MURPHY et al.[36] showed that the treatment of HuPBL/SCID mice with antibodies directed towards CD40 could improve secondary human IgG responses in these mice. HuPBL/SCID mice treated with anti-CD40 mAbs produced high human IgG responses to doses of diphtheria toxin (DT) vaccine challenge. In these mice however, there was little or no DT-specific IgM response detected, suggesting that anti-CD40 antibody stimulation in huPBL/SCID mice promotes strong human secondary IgG responses[36]. These data suggest that the use of anti-CD40 is selective in inhibiting transformed cells but promoting normal B cell function.

Treatment with anti-CD40 antibodies also increased

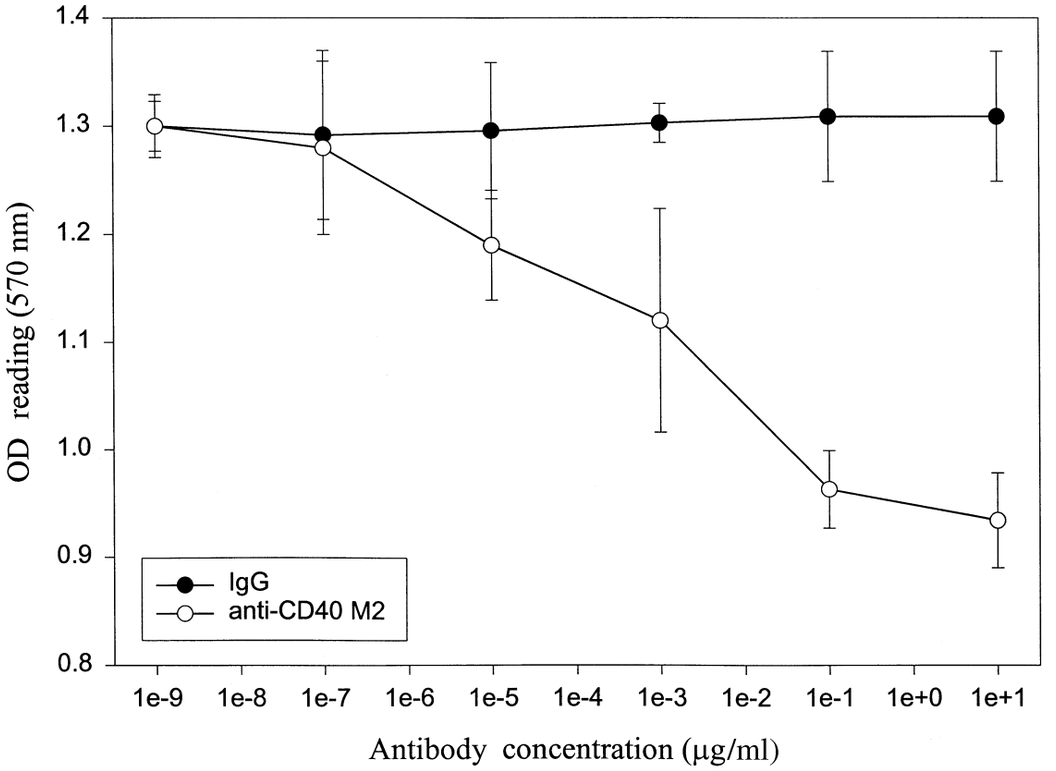

Fig. 1. Effects of anti-CD40 antibody on carcinoma proliferation. MDA231 cells were treated with anti-CD40 (M2 clone) and isotype matched IgG1 for 72 h. Proliferation was measured by MTT incorporation and optical density (OD) was taken at a wavelength of 570 nm. Greatest inhibition was seen at 10 μg/ml

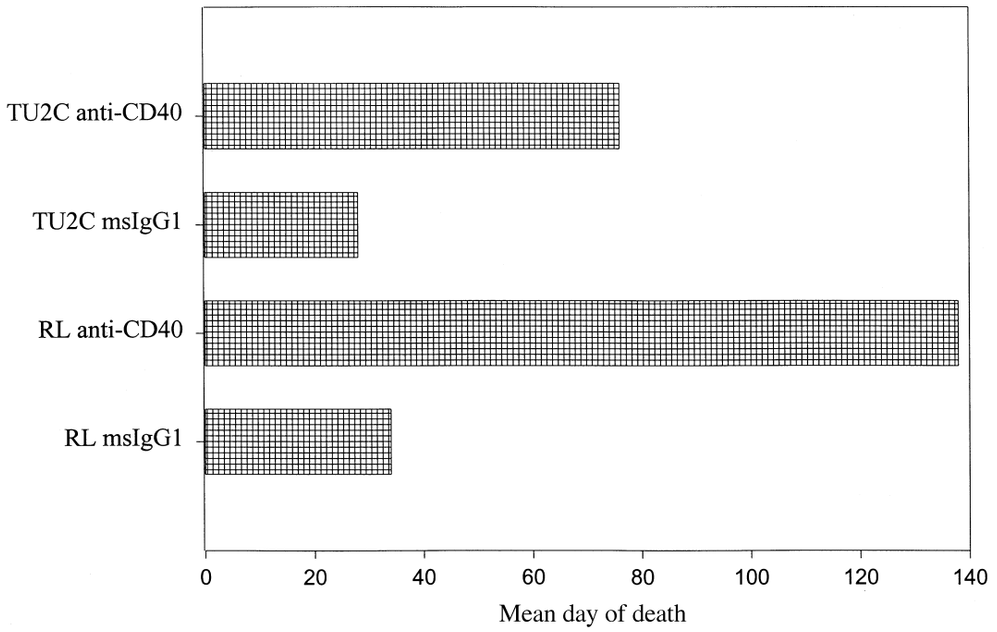

Fig. 2. Effects of anti-CD40 on survival of tumor-bearing mice. Mice were treated with either isotype matched control antibody or anti-CD40 (M2 clone). Two lymphoma lines were examined, RL and TU2C. Effects of the anti-CD40 antibody were measured by the mean day of death seen in each group of treated mice

the survival of mice-bearing human B lymphoma (Fig. 2). The inhibition of these B cell lymphomas *in vivo* appears to be due to AICD of the lymphoma cells, although antibody-dependent cell-mediated cytotoxicity (ADCC) also plays a role in the efficacy of these antibodies *in vivo*[15]. Similarly, increases in survival were seen in mice bearing carcinomas[24]. HIRANO et al.[24] showed that the use of anti-CD40 (M3 clone) in the treatment of tumor-bearing SCID mice was capable of significantly increasing the survival of these mice. In the same model, it was also seen that srhCD40L was able to significantly prolong the survival of human carcinoma-bearing mice.

Anti-CD40 Treatment and EBV Lymphomas

Treatment of B cells with anti-CD40 antibodies has been shown to prevent the occurrence of EBV-lymphomas *in vitro*[15]. FUNAKOSHI et al.[11] also showed that treatment of EBV transformed B cells with anti-CD40 inhibited growth of these cells. When HuPBL from EBV-seropositive donors is injected into SCID mice, B-lymphomas spontaneously arise[37]. These EBV-induced lymphomas are analogous to those found in immunocompromised individuals such as AIDS patients or organ-transplant recipients[46]. FUNAKOSHI et al.[11] also demonstrated that the incubation of anti-CD40 with EBV-induced B-lymphoma cell lines derived from HuPBL/SCID mice resulted in a significant inhibition of proliferation *in vitro* and *in vivo*.

Treatment of SCID mice receiving huPBL with anti-CD40 mAbs has also been shown to prevent the occurrence of EBV-induced B-lymphomas. The administration of anti-CD40 mAbs at the time of huPBL transfer into SCID mice not only prevented EBV lymphoma development, but also promoted normal human B cell engraftment in the recipient mouse[11, 15]. Thus, CD40 stimulation augments normal human B cell function but inhibits neoplastic B cell growth.

Treatment with CD40L

Activation of CD40 by CD40L in five different aggressive histology NHL cell lines has been shown to have anti-proliferative effects on these lines that include an increase in Fas expression and, therefore, Fas-mediated apoptosis[53]. FUNAKOSHI et al. also showed that treatment of various B lymphomas with CD40L also had an inhibitory effect on proliferation *in vitro*. Incubation of Daudi lymphoma cells with CD40L produced a maximal inhibition of 60–80% (Fig. 3).

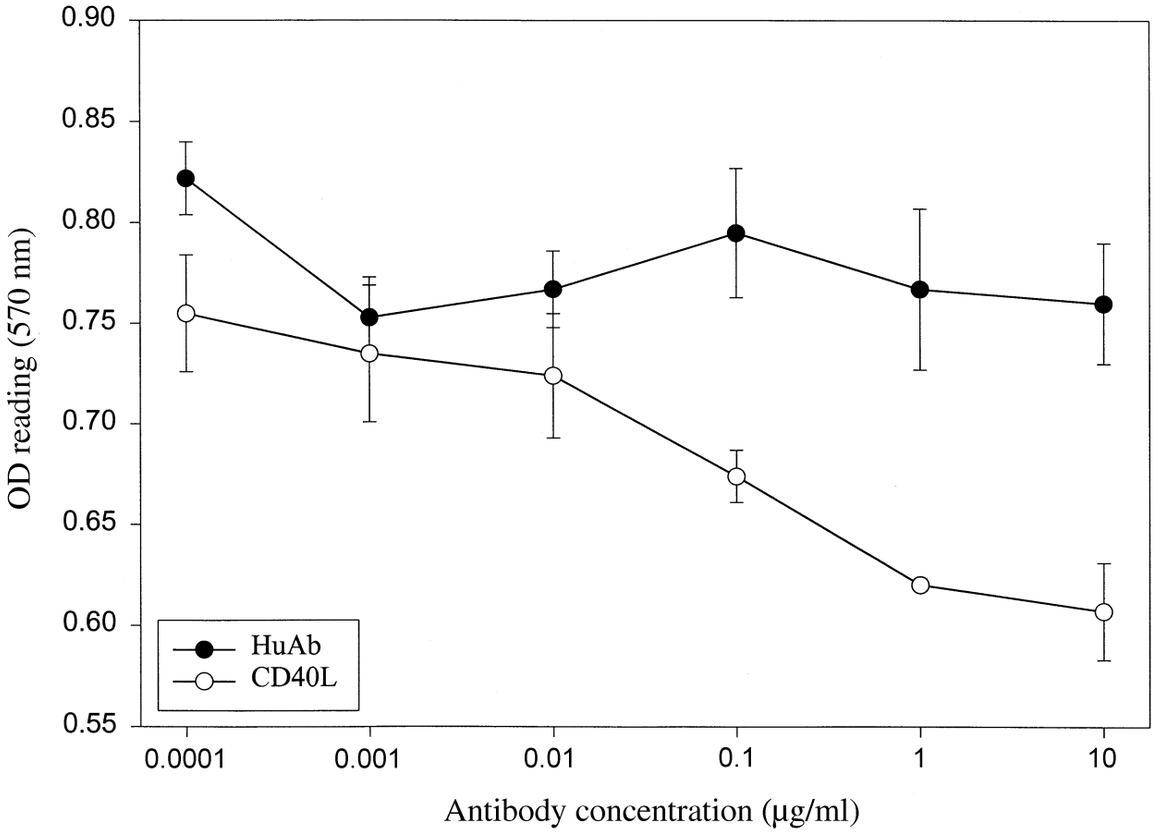

Fig. 3. Effects of srhCD40L on lymphoma proliferation. Daudi cells were treated with srhCD40L or human antibody (HuAb) serum for 72 h. Proliferation inhibition was measured by MTT incorporation and optical density (OD) was taken at a wavelength of 570 nm. The greatest inhibition of proliferation was seen at 10 μg/ml

Furthermore, stimulation of CD40 by CD40L has been shown to be beneficial in a syngeneic bone marrow transplantation model[14]. FUNAKOSHI et al.[14] showed that in lethally irradiated mice, treatment with a recombinant soluble murine CD40L following syngeneic bone marrow transplant resulted in increased bone marrow and splenic hematopoietic progenitor cells. Treatment with CD40L also resulted in an increase in platelet and granulocyte recovery in the peripheral blood, indicating that CD40L may be advantageous in facilitating hematopoietic reconstitution[14]. Furthermore, there was also an increase in T cell mitogen responsiveness and an increased production of IL-4[15]. B cell recovery was also accelerated in this model[14].

CD40 stimulation by a recombinant soluble human CD40L has also been shown to significantly inhibit the proliferation of human breast carcinoma as well as inducing apoptosis in the $CD40^+$ carcinoma lines tested (Fig. 4). WINGETT et al.[52] showed that CD40L signaling in IFN-γ pretreated MDA231 and T47D cells significantly decreased proliferation. Apoptosis was not observed in T47D cells with CD40L alone, showing the necessity of IFN-γ treatment to upregulate CD40 expression. The addition of anti-Fas antibody along with CD40L treatment produced higher levels of apoptosis, denoting a synergy between the two.

In conclusion, CD40 stimulation by its ligand may give rise to two different outcomes. In untransformed B cells and low grade lymphomas such as follicular lymphomas, treatment with CD40L may result in increased growth, whereas in aggressive B lymphomas, CD40L treatment appears to result in activation induced cell death. In breast carcinoma cells, however, CD40 ligation only appears to result in growth inhibitory or apoptotic effects. Cytotoxicity may be augmented by the addition of IFN-γ, which increases CD40 expression on these cells, therefore increasing the anti-proliferative effects of CD40L.

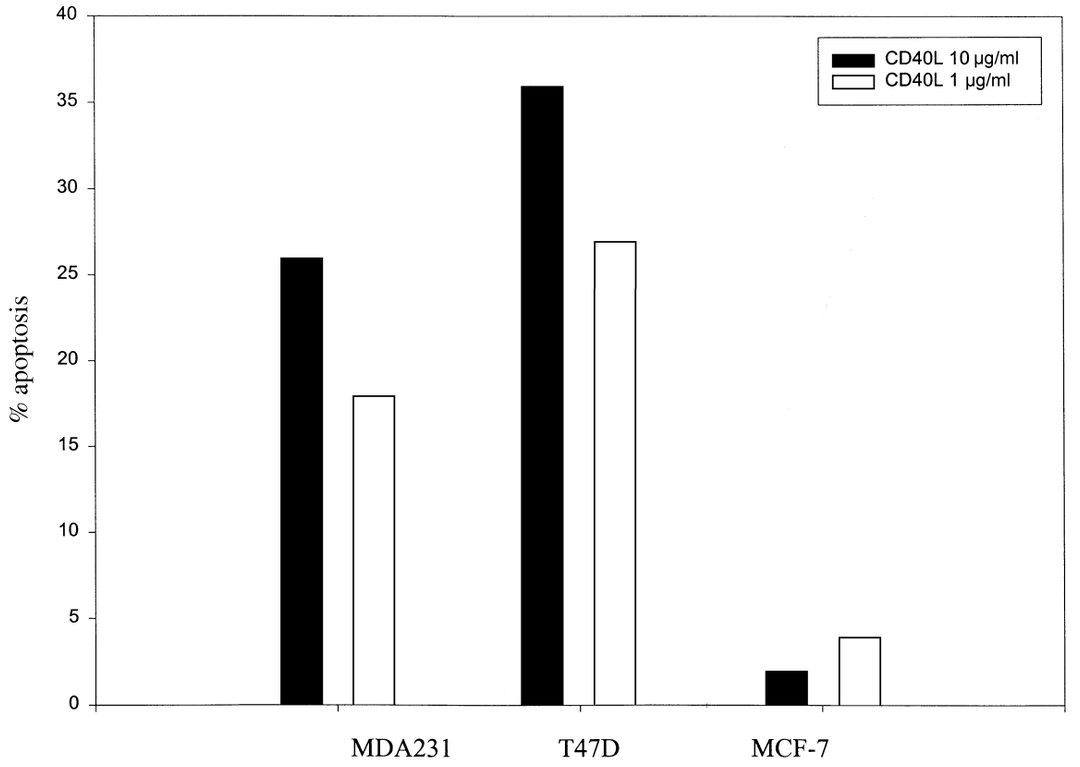

Fig. 4. Effects of CD40L on breast carcinoma lines. Human breast carcinoma lines MDA231, T47D and MCF-7 were incubated for 72 h with 10 µg/ml and 1 µg/ml CD40L. Apoptosis levels were assessed by nuclear matrix protein levels in the culture supernatant using an enzyme-linked immunosorbent assay kit

Clinical Applications

One major factor affecting the use of mAbs in treatment is the neutralizing human anti-mouse antibodies (HAMA) that often result during antibody treatment. Anti-CD40 inhibitory effects primarily appear to be due to ADCC[13]. The use of soluble CD40L can circumvent many of the problems associated with the use of antibodies for treatment. The leucine zipper motif on CD40L has little or no immunogenic effect[3].

The mechanism by which CD40 is able to eradicate transformed cells is yet unknown. It is possible that there is exogenous production of TNF upon CD40 stimulation, therefore causing involvement by TNFR-1 by CD40 stimulated cells. Another possible mechanism may be due to an increase in Fas expression on some CD40 stimulated cells. Finally, it is possible that CD40 stimulation may increase levels of pro-apoptotic genes and proteins such as Bax and Bclx-S. However, additional studies are required to determine the precise mechanisms of CD40s anti-tumor responses as well as the types of cancer in which CD40 stimulation might be clinically useful.

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Received in October 1999
Accepted in February 2000